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Purification of Myogenic Progenitors from Human Muscle Using Fluorescence-Activated Cell Sorting (FACS)

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Abstract

Primary myoblasts derived from human tissue are a valuable tool in research of muscle disease and pathophysiology. However, skeletal muscle biopsies, especially from diseased muscle, contain a plethora of non-myogenic cells, necessitating purification of the myogenic cell population. This protocol describes techniques for dissociation of cells from human skeletal muscle biopsies and enrichment for a highly myogenic population by fluorescence-activated cell sorting (FACS). We also describe methods for assessing myogenicity and population expansion for subsequent in vitro study.

Keywords

Skeletal muscle; Myoblast isolation; Tissue dissociation; Fluorescence-activated cell sorting (FACS); CD82; CD56; Immunostaining; Pax7

1 Introduction

Myogenic cells derived from muscle biopsies are a valuable resource for modeling human muscle disease in vitro. They can be utilized to assess many cell-based and pharmacological therapies for translational research by assaying myoblast proliferation, differentiation, and fusion, which are often compromised in diseased states. However, human skeletal muscle biopsies, especially those affected by disease, often contain extensive populations of non-myogenic cells including adipocytes and fibroblasts that may confound experimental results. Thus, it is important to isolate a myogenic population for accurate in vitro study of skeletal muscle development and disease.

Early studies of muscle disease through the 1970s largely involved the use of tissue explants or unpurified dissociated cells [1–4], which were used to determine in vitro conditions for culture and differentiation of human muscle cells [5, 6]. In the 1980s, Blau and Webster introduced a pre-plating technique to remove fibro-blasts [7], and this group later described more specific and efficient isolation of myoblasts from a dissociated human sample utilizing human neural cell adhesion molecule (NCAM), also known as CD56 [8], a cell surface

antigen shown to be expressed on myogenic cells [9] using fluorescence-activated cell sorting (FACS).

Here, we describe an effective technique for dissociation of mononuclear cells from human muscle biopsies, and purification of a highly myogenic population utilizing FACS to detect the cell surface markers CD56 in combination with tetraspanin CD82 (*see Note 1*). We recently demonstrated that CD82 is an excellent myogenic marker in both human fetal and adult skeletal muscle that is also retained on activated and differentiating myogenic progenitors [10]. This protocol also describes methods to confirm enrichment of muscle stem cells via Pax7 immunostaining; to culture the purified progenitors and confirm the presence of a myogenic population with an in vitro fusion assay. Isolation and expansion of these cells from normal individuals and from individuals with muscle disorders will help accelerate the development of therapies for human disorders such as muscular dystrophies.

2 Materials

2.1 Dissociation of Primary Cells

1. Protected disposable scalpels with stainless steel blade size #10.
2. Sterile 10 cm tissue culture-treated plastic dishes.
3. Assorted sterile 5, 10, and 25 mL pipettes.
4. Sterile 0.22 μ m Polyethersulfone (PES) low protein binding membrane filters, 250 and 500 mL volumes.
5. 0.22 μ m Cellulose nitrate (CN) filter unit, 500 mL volume.
6. Sterile 15 and 50 mL conical centrifuge tubes.
7. BD Falcon sterile nylon cell strainers, 100 μ m and 40 μ m pore sizes.
8. 10 $\times$ Hank's Balanced Saline Solution (HBSS), free of calcium chloride, magnesium chloride, and magnesium sulfate, diluted to 1 $\times$ with double distilled water and filter sterilized with a 0.22 μ m CN filter. This solution can be stored at 4 $^{\circ}$ C or room temperature.
9. Complete growth medium: High glucose Dulbecco's Modified Eagle's Medium (DMEM), 20% fetal bovine serum (FBS), 1% Penicillin-Streptomycin-Glutamine (PSG 100 $\times$ stock, Thermo Fisher 10378016). Mix 395 mL of high glucose DMEM with 100 mL of FBS (*see Note 2*) and 5 mL of 100 $\times$ PSG. Sterilize by filtering the solution through a 500 mL 0.22 μ m PES filter unit. Store at 4 $^{\circ}$ C and use within 1 month.
10. Sterile HEPES buffered saline solution, without phenol red.
11. 1M calcium chloride solution ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, FW 147). Dissolve 1.47 g powder in 10 mL of double distilled water. Store at 4 $^{\circ}$ C.
12. Dispase II 2.4 U/mL stock solution. Dissolve 1 g powder dispase II (Roche Applied Science) in 100 mL HEPES buffered saline to generate a stock solution of 2.4 U/mL. Check the total units for the specific dispase lot and add the

appropriate volume of DMEM to reach the 2.4 U/mL concentration. Filter-sterilize the solution through a PES 500 mL filter; aliquot into 15 mL conical tubes (10 mL/tube) and store aliquots at -20°C .

13. Collagenase D stock solution: dissolve 2.5 g powder collagenase D (Roche Applied Science) in 250 mL solution of $1\times$ HBSS supplemented with 1.25 mL of 1 M CaCl_2 . Sterilize by filtering through a PES filter unit. The filtered solution can be dispensed in 15 mL conical tubes (10 mL/tube) and stored at -20°C .
14. Sterile freezing medium: 90% FBS and 10% dimethyl sulfoxide (DMSO). Prepare freezing medium and immediately store on ice. Unused sterile freezing medium can be stored at 4°C for up to 4 weeks.
15. Sterile red blood cell lysis solution, stored at room temperature (such as Qiagen RBC Lysis solution).
16. Sterile 1.8 mL CryoTube™ vials.
17. Bench top centrifuge.
18. Hemocytometer.
19. Sterile laminar flow biosafety cabinet.
20. -150°C freezer, liquid nitrogen storage tank.
21. Humidified 5% CO_2 incubator set to 37°C .

2.2 Purification of Myoblasts

2.2.1 Thawing of Cryopreserved Sample

1. Sterile 0.22 μm PES filter, 500 mL volume.
2. Sterile 50 mL conical centrifuge tubes.
3. Sterile tissue culture-treated plastic dishes, 10 or 15 cm size.
4. CryoTube™ vial containing dissociated unpurified primary cells.
5. Sterile complete growth medium, as above.
6. Sterile laminar flow biosafety cabinet.
7. Water bath set to 37°C .
8. CO_2 incubator, as above.

2.2.2 Preparation of Sample for FACS

1. Sterile 0.22 μm PES filter, 50 mL volume.
2. Sterile 15 and 50 mL conical centrifuge tubes.
3. Sterile 5 mL round bottom test tubes with cell strainer caps.
4. Dissociated unpurified primary cells.
5. Sterile $1\times$ HBSS (diluted from $10\times$ stock).

6. Sterile 1× Dulbecco's Phosphate Buffered Saline (DPBS, diluted from 10× stock).
7. TrypLE Express™ dissociation enzyme with phenol red.
8. Antibodies (all stored at 4 °C, protected from light): APC anti-CD56 antibody, Clone HCD56 (BioLegend, catalog number: 318310); PE anti-CD82 antibody, Clone ASL-24 (BioLegend, catalog number: 342103).
9. Calcein blue (1 mg vial): Resuspend in 200 µL dimethyl sulf-oxide (DMSO). Aliquot in 25 µL aliquots and store at −20 °C (stock). Use 0.5 µL stock calcein/10⁶ cells.
10. Sterile 0.5% Bovine Serum Albumin (BSA) in HBSS. Add 2.5 g BSA to 1 HBSS. Sterilize by filtering the solution through a 500 mL 0.22 µm PES filter unit. Store at 4 °C.
11. Sterile laminar flow biosafety cabinet.
12. Bench top centrifuge.
13. Inverted microscope.
14. Hemocytometer.

2.2.3 Fluorescence-Activated Cell Sorting (FACS)

1. FACS or 5 mL round-bottom tubes.
2. Cell sorting machine.
3. Cell sorting software.

2.3 In Vitro Culture of Myoblasts

2.3.1 In Vitro Cell Culture

1. Sterile 50 mL conical centrifuge tubes.
2. Sterile 10 cm tissue culture-treated plastic dishes.
3. Sterile 0.22 µm PES filter, 50 mL volume.
4. Sterile 1× DPBS.
5. TrypLE™ Express Dissociation Enzyme with Phenol Red (Invitrogen).
6. Sterile complete growth medium, as above.
7. Differentiation medium (50 mL): Mix 48.5 mL of low glucose Dulbecco's Modified Eagle's Medium (DMEM) with 1 mL of horse serum (HS) and 0.5 mL of 100× Penicillin-Streptomycin-Glutamine (PSG). Sterilize by filtering the solution through a 150 mL 0.22 µm PES filter unit. Store at 4 °C and use within 1 month.
8. 0.1% gelatin: Add 0.5 g gelatin to 500 mL of double distilled water, do not shake. Sterilize the solution by autoclaving for 20 min and store at 4 °C.

9. Sterile laminar flow biosafety cabinet.
10. Water bath set to 37 °C.
11. Humidified 5% CO₂ incubator set to 37 °C.
12. Bench top centrifuge.
13. Inverted microscope.
14. Hemocytometer.

2.3.2 Immunofluorescent Staining of Pax7 and In Vitro Fusion Assay

1. Aluminum foil.
2. Microscope slides, 25 × 75 mm, 1 mm thick, such as Tissue Tack microscope slides from Polysciences.
3. Cytospin funnels, such as EZ Single Cytofunnel (Shandon).
4. Cytospin centrifuge, such as Cytospin 4 (Thermo Shandon).
5. 4-well chamber slides, Nunc Lab-Tek II Permanox.
6. 10× Phosphate Buffered Saline (PBS), diluted to 1× with double distilled water. Store at room temperature.
7. 4% paraformaldehyde (4% PFA) in PBS. Dilute 16% paraformaldehyde with 1× PBS. USE CAUTION as paraformaldehyde is extremely toxic; it is recommended that paraformaldehyde be used in a fume hood for safety. Aliquot and store at −20 °C. Aliquots should not be repeatedly frozen and thawed; discard unused PFA after initial use.
8. Permeabilization solution: PBS 0.5% Triton-X100. Mix 50 µL of Triton X-100 with 10 mL of 1× PBS.
9. Blocking solution: 10%FBS, 0.1% Triton-X100 in PBS. Mix 1 mL of fetal bovine serum (FBS), 10 µL of Triton X-100, and 9 mL of 1× PBS.
10. Antibodies (all stored at 4 °C): Anti-Pax7 (Developmental Studies Hybridoma Bank, PAX7 concentrate supernatant); AffiniPure F(Ab')₂ Alexa Fluor 594 Donkey anti-Mouse IgG (H + L) (Jackson ImmunoResearch, catalog number: 715–586-150), protect from light.
11. Inverted microscope with epi-fluorescence capabilities including ultraviolet/DAPI, FITC/GFP and Rhodamine/TRITC filter sets.

3 Methods

3.1 Dissociation of Primary Cells (See Note 3)

All the steps in this protocol should be performed in a sterile laminar flow biosafety cabinet using the sterile tissue culture technique. Human skeletal muscle can only be obtained

following approval from the Institutional IRB. We obtained de-identified, discarded skeletal muscle tissue under a protocol approved by Boston Children's Hospital IRB.

1. Pre-weigh one 10 cm tissue culture plate, and place the tissue sample to be dissociated in a second (non pre-weighed) 10 cm tissue culture plate.
2. Using sterile scalpels, remove any connective tissue from the muscle tissue. Tissue should be kept moist. Add a few drops of sterile 1× HBSS to tissue as necessary to prevent it from drying out. Place muscle tissue in the pre-weighed 10 cm tissue culture plate, replace the lid, and weigh the plate again. Subtract from this number the tare of the empty plate to calculate the amount of muscle tissue to be dissociated.
3. Thaw frozen aliquots of dispase II and collagenase D in a 37 °C water bath. The solutions will be added at a volume of 3.5 mL each per gram of muscle tissue to be dissociated. Thaw only the amounts of collagenase D and dispase II necessary for dissociation. If an excess of enzymes is thawed, it can be refrozen once and reused.
4. Using sterile scalpels, mince muscle tissue until it resembles a fine paste. During mincing, add a few drops of sterile 1× HBSS to prevent exposed tissue from drying out. Tissue should always appear moist, but with no excess of liquid.
5. After tissue is finely minced, add equal amounts of the thawed dispase II and collagenase D solutions. The final concentration will be 5 mg/mL for collagenase D and 1.2 U/mL for dispase II in this solution. Pipette minced tissue and enzyme solution up and down through a sterile wide-bore 25 mL pipette a few times.
6. Incubate the plate in a humidified 5% CO₂ incubator set to 37 °C for 15 min.
7. Pipette the digestion solution up and down through a sterile 25 mL pipette a few times and incubate again for 15 min. Repeat this step an additional 1–2 times, until the slurry easily passes through a sterile 5 mL pipette and all tissue chunks are dissolved. The total digestion time will range between 45 min and 1 h 15 min.
8. Add 2 volumes of complete growth medium (based on the total volume of dispase II and collagenase D) to the digested slurry and filter the digestion solution through a 100 µm cell strainer over a 50 mL conical tube. Change cell strainer if it appears clogged.
9. Pellet cells for 10 min at 1100 × *g*, room temperature.
10. Resuspend the pellet in 1 volume of complete growth medium (i.e., 3 mL) and add 7 volumes (i.e., 21 mL) of red blood cell lysis solution. Invert the tube a few times and then filter the solution through a 40 µm cell strainer over a 50 mL conical tube.
11. Count cells using a hemocytometer, then pellet the cells for 10 min at 1100 × *g*, room temperature. Expect approximately 10⁷ cells/gram tissue from postnatal

skeletal muscle and 10^8 cells/gram tissue from fetal skeletal muscle. Cell numbers vary among individuals.

12. Freeze cells at a concentration of 1×10^7 cells/mL in ice-cold freezing medium. Store cryovials at -80°C overnight, then transfer them to -150°C where they can be permanently stored until necessary. Cell freezing is not required if all reagents and FACS equipment are immediately available. In this case, proceed to Subheading 3.2.2, **step 11**.

3.2 Purification of Myoblasts

All the steps in this protocol except for cell sorting (Subheading 3.2.3) should be performed in a sterile laminar flow hood using the sterile tissue culture technique. Cell sorting should be performed in as clean an environment as possible.

3.2.1 Thawing of Cryopreserved Sample Prior to FACS—Cryopreserved cells should be carefully thawed and plated 1 day prior to cell sorting. This allows the cells to recover from the freezing process before undergoing FACS.

1. Pre-warm complete growth medium in a water bath set to 37°C . Then, pipette 10 mL pre-warmed medium into a sterile 50 mL conical tube.
2. Coat sterile tissue culture-treated plates (10 cm plate) with 10 mL 0.1% gelatin for 1 h at 37°C , then remove the gelatin solution by aspiration.
3. Let the plates dry briefly in the biosafety cabinets and replace the lid.
4. Carefully and quickly thaw a vial of cryopreserved, dissociated cells in a 37°C water bath and transfer the cells into the 50 mL conical tube with 15 mL pre-warmed proliferation medium using a 1 mL pipette. Rinse the inside of the cryovial with fresh complete growth medium to remove as many cells as possible. This step should be performed very quickly as the DMSO used during the cryopreservation process is toxic to the cells at room temperature.
5. Plate the cells in the pre-warmed medium onto sterile, tissue-culture treated plates at approximately $0.5\text{--}1 \times 10^7$ cells/10 cm plate or $1.5\text{--}3 \times 10^7$ cells/15 cm plate. If using a 15 cm plate, add 15 mL pre-warmed medium to bring the total medium volume to 25 mL.
6. Incubate the cells in a humidified 5% CO_2 incubator set to 37°C overnight. If plating cells several days in advance, change the growth medium every other day and do not allow confluence to exceed 80%.

3.2.2 Preparation of Sample for FACS

1. Pre-warm complete growth medium and $1 \times$ DPBS in a 37°C water bath. Place the 0.5% BSA/HBSS on ice.
2. Check cells under a phase contrast microscope with $10\times$ magnification (*see* Note 4). Ensure that there is no contamination and that the cells look healthy.

3. Wash the cells with 5 mL (10 cm plate) or 10 mL (15 cm plate) of 1× DPBS 2–3 times.
4. Pipette 2 mL (10 cm plate) or 5 mL (15 cm plate) TrypLE Express™ dissociation enzyme onto the plate of washed cells and incubate in a humidified 5% CO₂ incubator set to 37 °C for 2 min.
5. Check under the microscope if the cells have lifted and are now floating freely in the medium. If the cells still adhere to the plate, gently tap the bottom of the plate to loosen the cells and return the plate to the incubator for another minute.
6. After incubation, remove the cells by gently swirling the medium, pipetting the cells a few times and pooling the cells to one side of the plate by tilting it at an angle (~45°), then carefully pipette the medium into the 50 mL conical tube (*see* Note 5).
7. Repeat **step 6** with growth medium (5 mL for 10 cm plate and 10 mL for 15 cm plate) to collect any remaining cells and quench the TrypLE™ (*see* >Note 6).
8. Check the plate under a phase contrast microscope at 10× magnification for the presence of cells. There should be very few cells on the surface of the plate after this process.
9. Centrifuge the 50 mL conical tubes containing the cells and wash at 1100 × *g* at 4 °C for 10 min to pellet the cells.
10. Remove the supernatant, and resuspend the cells in 10 mL 5% FBS/HBSS.
11. Determine the cell concentration using a hemocytometer or other cell counting device.
12. For FACS controls, use 5 mL round-bottom test tubes and set aside 2.5×10^5 cells in 500 µL 5% FBS/HBSS for each of the following controls: “Unstained” control; Calcein Blue single color control (live cells); CD56 single color control; CD82 single color control.
13. Pipette the “unstained” control sample through the strainer cap of a 5 mL round-bottom test tube (*see* Note 7). Keep on ice.
14. Centrifuge the remaining cells (to be labeled with both CD56 and CD82 antibodies or single color controls) for 10 min at 1100 × *g* at 4 °C.
15. Resuspend cells at a concentration of 1×10^7 /mL in 5% FBS/HBSS.
16. Primary antibody incubation: add CD56 and CD82 antibodies to the appropriate cell solutions at a concentration of 5 µL per 1×10^6 cells (as recommended by the manufacturer).
17. To gate for live cells, add calcein blue at a concentration of 0.5 µL per 1×10^6 cells to the appropriate cell solutions. Gently mix and place on ice protected from the light for 30 min (*see* Note 8).
18. After this incubation, wash the cells 1× in 2 mL of 5% FBS/HBSS.

19. Centrifuge the cells for 10 min at $1100 \times g$ at 4 °C.
20. Resuspend the CD56 and CD82 single color controls in 500 μ L of 5% FBS/HBSS, and pipette through the strainer cap of a 5 mL round bottom test tube. Store on ice in the dark.
21. Resuspend the CD56/CD82/calcein blue stained cells in 1 mL of 5% FBS/HBSS, and pipette through the strainer cap of a 5 mL round-bottom test tube. Store on ice in the dark.
22. Prepare collection tube for CD56⁺CD82⁺ sorted cells by pipetting 500 μ L of growth medium into a new tube. Store on ice.

3.2.3 Fluorescence-Activated Cell Sorting—It is beyond the scope of this chapter to review FACS or flow cytometry in detail. Gating specifications are briefly indicated.

1. Determine optimal excitation voltages and compensation values using the “no stain” and single color controls (Fig. 1a).
2. Determine the live cell population gating for calcein blue positive cells (Fig. 1b).
3. Determine the double positive (DP) CD56⁺/CD82⁺ and double negative (DN) populations. Gate and sort for the DP cell population (Fig. 1c).

3.3 In Vitro Culture of Myoblasts

3.3.1 In Vitro Cell Culture—All the steps in this protocol except immunofluorescent staining (Subheading 3.3.5) should be performed in a sterile laminar flow hood using the sterile tissue culture technique.

1. Coat sterile 10 cm tissue culture-treated plates with 10 mL 0.1% gelatin for 1 h in a humidified 5% CO₂ incubator set to 37 °C, then remove the gelatin solution by aspiration. Let the plates dry briefly in the biosafety cabinets and replace the lid.
2. Pre-warm complete growth medium in a water bath set to 37 °C.
3. Resuspend sorted CD56/CD82 double positive cells at $0.5\text{--}1 \times 10^6$ cells/10 mL complete growth medium and plate on coated plates. Gently rock plate(s) to evenly distribute cells, and then place in a 5% CO₂ incubator set to 37 °C. Sorted cells will be small and have a bright, rounded appearance and should attach within 1 day post-sorting.
4. Propagate the cells to 60–75% confluency (*see* Note 9). This should take approximately 2–3 days; however, if necessary, replace the medium with fresh growth medium every 2 days until the plate is at 60–75% confluency.

3.3.2 Cell Passaging

1. Coat sterile tissue culture-treated plates with 0.1% gelatin as in Subheading 3.3.1, **step 1**.

2. Pre-warm the following in a water bath set to 37 °C: 1× DPBS, TrypLE™ Express dissociation enzyme, and complete growth medium.
3. Remove the medium from the plate by aspiration and wash the cells twice with 10 mL (10 cm plate) 1× DPBS. Remove DPBS by aspiration.
4. Pipette 2 mL TrypLE™ Express onto the plate and incubate in a humidified 37 °C CO₂ incubator for 2–3 min. Gently remove the cells from the plate by pipetting up and down a few times before transferring cells into a sterile conical tube. Wash any remaining cells from the surface of the plate with additional complete growth medium.
5. Centrifuge the cells at 1100 × *g* at room temperature for 10 min.
6. Resuspend the cells in 10 mL fresh complete growth medium.
7. Determine the cell concentration using a hemocytometer and plate the cells at 0.5–1 × 10⁶ cells in 10 mL complete growth medium/10 cm plate.
8. Cells should be passaged every 2–3 days and should not be grown past 75% confluency.

3.3.3 Cell Freezing

1. Remove the medium from the plate by aspiration and wash the cells twice with 10 mL (10 cm plate) 1× DPBS. Remove DPBS by aspiration.
2. Pipette 2 mL TrypLE™ Express onto the plate and incubate in a humidified 5% CO₂ incubator set to 37 °C for 2–3 min.
3. Gently remove the cells from the plate by pipetting up and down a few times before transferring cells into a sterile conical tube. Wash any remaining cells from the surface of the plate with additional complete growth medium.
4. Centrifuge the cells at 1100 × *g* at room temperature for 10 min.
5. Resuspend cells in ice-cold freezing medium (10% DMSO in 90% fetal Bovine Serum) at desired cell concentration (10⁶–10⁷/mL).
6. Store cryovials at –80 °C overnight and then transfer to –150 °C where they can be permanently stored until necessary.

3.3.4 In Vitro Fusion Assay

1. Coat 4-well chamber slides with 0.1% gelatin.
2. Trypsinize the cells with TrypLE Express™ Dissociation Enzyme with Phenol Red and determine the cell concentration as described above, then plates 20,000 cells in 500 µL complete growth medium/well.
3. Incubate the cells in a humidified 5% CO₂ incubator set to 37 °C until the cells are ~80% confluent.
4. When the cells are ~80% confluent, remove the growth medium from each well and replace with 500 µL pre-warmed differentiation medium (*see* Note 10).

5. Incubate the cells in a humidified 5% CO₂ incubator set to 37 °C overnight.
6. Replace the differentiation medium in each well daily during the course of the fusion assay.
7. Monitor the differentiation of the cells using a phase contrast microscope at 10× or 20× magnification. Fusion and robust formation of myotubes should occur within 1 week of exposure to differentiation medium (*see examples in Fig. 2*).

3.3.5 Immunofluorescent Staining for PAX7 (See Note 11)

1. Thaw 4% PFA at room temperature.
2. Following cell sorting, set aside an aliquot of the sorted cells for cytopins. Estimate to use between 2000 and 3000 cells for each cytopsin.
3. Resuspend the cells for cytopins at a concentration of 2000 cells/150 µL in 4% PFA solution. Fix cells for 20 min at room temperature; then cytopins 150 µL cells in each cytofunnel. Spin at 600 rpm for 5 min, then remove slides, draw a hydrophobic barrier around the cytopsin cells, and place the slides in 1× PBS (*see Note 12*).
4. Permeabilize the cells with 200 µL of permeabilization solution for 3 min at room temperature.
5. Remove permeabilization solution using a pipette, and then block the cells for 30 min at room temperature with 200 µL of blocking solution.
6. Prepare the primary antibody solution by diluting the anti-PAX7 concentrate supernatant antibody 1:100 in fresh blocking solution. Incubate the cells with primary antibody solution overnight at 4 °C.
7. Wash the cells three times with 1× PBS for 5 min at room temperature. The slides may be gently agitated on a rotating shaker.
8. Prepare the secondary antibody solution by diluting the anti-mouse antibody 1:1000 in blocking solution. Incubate the cells in the dark with secondary antibody solution for 1 h at room temperature.
9. Wash the cells three times with 1× PBS for 5 min at room temperature in the dark. The plate may be gently agitated on a rotating shaker.
10. Mount the cells with Vectashield with DAPI and store at 4 °C protected from light with aluminum foil.
11. Visualize the cells by fluorescent microscopy using ultraviolet/DAPI and TRITC filter sets for DAPI and PAX7, respectively. Following cell sorting >85% of the cells should be expressing PAX7 when using fetal tissue (*see Fig. 3*).

3.3.6 In Vitro Fusion Assay Fusion Index Calculation

1. Count the following in each of five random fields per well: Number of total nuclei; number of nuclei within myotubes..

2. Calculate fusion index (%) as: number of nuclei within myo-tubes/number total nuclei ($\times 100$).
3. Average the fusion index of the five fields.
4. Compare the fusion index of sorted myoblasts versus unsorted.

4 Notes

1. We would like to note that FACS with either CD56 (NCAM) or CD146 (MCAM) in conjunction with CD82 as enriching markers is a highly effective method for isolating human fetal myogenic progenitors. We refer the readers to the following protocol describing use of MCAM as a positive selection marker in cells sorted from human fetal tissue [11]. For adult skeletal muscle, CD82 should be used in conjunction with CD56, since endothelial cells express MCAM.
2. FBS varies considerably between companies and even lot to lot from the same company. Therefore, several different FBS samples should be tested using the in vitro methods described in Subheading 3.3 to determine which lot/company works best.
3. Institutional review and protocol approval are required prior to collection and processing of human tissue. All personnel handling human tissue must receive appropriate safety and human subject education training.
4. There will be many floating, live cells in the culture, which is normal for dissociated human skeletal muscle. It is also likely that there will be small clumps of cells in the culture, and the number of clumps will vary. These clumps will be filtered out prior to cell sorting. Additionally, the dissociation process results in a large amount of debris in addition to cells. This will make the culture appear “dirty” (i.e., little black specks, etc.), but again, this is normal and should not be considered contamination. This debris will be removed during the FACS sample preparation process.
5. When the plate is tilted at an angle, the cells can be seen on the surface of the plate as a light opaque coating. Repeatedly rinse gently the cells off the plate using the trypsin solution (TrypLE Express™ Dissociation Enzyme with Phenol Red) until this coating is no longer visible.
6. Prolonged exposure of the cells to undiluted TrypLE Express™ may negatively affect the health of the cells. Addition of growth medium to the trypsinized cells will quench this effect.
7. Some FACS machines may require tubes that are different in diameter/size from the tube specified in this protocol. Check in advance that the tubes fit in the FACS machine.
8. Calcein blue is a cell viability dye and is used in this protocol to discriminate live from dead cells/debris during the FACS.

9. Cells should never reach 100% confluency when proliferating, as they will begin to differentiate and fuse on contact. The high serum growth medium will lower in serum concentration over time and will not be able to prevent fusion (*see* Note 10).
10. Low serum medium induces differentiation and fusion of myoblasts in culture [12].
11. This immunofluorescent staining protocol can also be utilized for the detection of other myogenic markers of proliferating or differentiating cells.
12. It is useful to draw a hydrophobic barrier around the spot where the cells were deposited on the slide following cytopins using a PAP pen. This will minimize the amount of antibody solution to be used during the immunostaining process.

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Fig. 1. Gating of myogenic cells double positive for CD56 and CD82 from dissociated human skeletal muscle following FACS analysis. **(a)** Unstained control; **(b)** Gating of live cells based on Calcein blue uptake and **(c)** gating of double positive cells (Q2) that will be sorted

Fig. 2. Examples of human fetal (**a, b**) and human adult (**c, d**) CD56⁺CD82⁺ sorted cells showing myotube formation. Scale bar: 100 μm. Arrows point to myotubes in the fields

Fig. 3. Immunostaining of CD82 and Pax7 in unpurified cells (upper panels) and in sorted CD56⁺ CD82⁺ progenitors (lower panels). CD82 is expressed in >90% of Pax7⁺ cells (arrowhead), although CD82⁺ Pax7⁻ cells are also present (arrows). Scale bars: 50 μm